Epigenetic Manipulation of Brain-derived Neurotrophic Factor Improves Memory Deficiency Induced by Neonatal Anesthesia in Rats

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ABSTRACT

Background: Although neonatal exposure to anesthetic drugs is associated with memory deficiency in rodent models and possibly in pediatric patients, the underlying mechanisms remain elusive. The authors tested their hypothesis that exposure of the developing brain to anesthesia triggers epigenetic modification, involving the enhanced interaction among transcription factors (histone deacetylase 2, methyl-cytosine-phosphate-guanine–binding protein 2, and DNA methyltransferase 1) in *Bdnf* promoter region(s) that inhibit brain-derived neurotrophic factor (BDNF) expression, resulting in insufficient drive for local translation of synaptic mRNAs. The authors further hypothesized that noninvasive environmental enrichment (EE) will attenuate anesthesia-induced epigenetic inhibition of BDNF signaling and memory loss in rodent models.

Methods: Seven days after birth (P7), neonatal rats were randomly assigned to receive either isoflurane anesthesia for 6 h or sham anesthesia. On P21, pups were weaned, and animals were randomly assigned to EE or a standard cage environment (no EE). Behavioral, molecular, and electrophysiological studies were performed on rats on P65.

Results: The authors found a substantial reduction of hippocampal BDNF (n = 6 to 7) resulting from the transcriptional factors–mediated epigenetic modification in the promoter region of *Bdnf* exon IV in rats exposed postnatally to anesthetic drugs. This BDNF reduction led to the insufficient drive for the synthesis of synaptic proteins (n = 6 to 8), thus contributing to the hippocampal synaptic (n = 8 to 11) and cognitive dysfunction (n = 10) induced by neonatal anesthesia. These effects were mitigated by the exposure to an enriched environment.

Conclusions: The findings of this study elucidated the epigenetic mechanism underlying memory deficiency induced by neonatal anesthesia and propose EE as a potential therapeutic approach. **(Anesthesiology 2016; 124:624-40)**

APPROXIMATELY 1.5 million infants and 4.5 million children undergo medical procedures that require general anesthesia each year in the United States,[1] and recent evidence indicates that young children exposed to even a single dose of anesthesia have an increased risk of neurocognitive dysfunction.[2] The developing brain undergoes significant cerebral plasticity, synapse formation, and maturation.[3] Virtually all anesthetics (isoflurane, propofol, nitrous oxide, midazolam, and ketamine) that act as γ-aminobutyric acid type A receptor agonists or *N*-methyl-D-aspartate glutamate receptor antagonists, alone or in combination, are implicated in long-term developmental neurotoxicity and cognitive deficits in animals and possibly in humans who had neonatal anesthesia.[4–7] For example, repeated exposure to propofol significantly impaired the induction of hippocampal long-term potentiation (LTP) and spatial learning ability, which are potentially associated with the downregulation of α isoform of calcium/calmodulin-dependent protein kinase II (CaMKIIα) signaling, in the developing rat brain.[8] Postoperative cognitive dysfunction, including impaired reaction time, psychomotor control, verbal recall, visual memory, and recognition memory, has been observed in pediatric patients receiving anesthesia with propofol or isoflurane.[9] Although previous studies reported the existence of extensive apoptotic neurodegeneration,[4,10] protracted injury to mitochondria,[6]

What We Already Know about This Topic

- Although neonatal exposure to anesthetic drugs is associated with memory deficiency in rodent models and possibly in pediatric patients, the underlying mechanisms remain elusive. Here, the authors tested the hypothesis that exposure of the developing brain to anesthesia trigger epigenetic modifications that inhibit brain-derived neurotrophic factor expression.

What This Article Tells Us That Is New

- The authors found a substantial reduction of hippocampal brain-derived neurotrophic factor resulting from the transcriptional factors–mediated epigenetic modification in the promoter region of *Bdnf* exon IV in rats exposed postnatally to anesthetic drugs. This brain-derived neurotrophic factor reduction led to the insufficient drive for the synthesis of synaptic proteins, thus contributing to the hippocampal synaptic and cognitive dysfunction induced by neonatal anesthesia. These effects were mitigated by the exposure to an enriched environment.

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and sustained neuroinflammation[11] in the brain of rats that underwent neonatal anesthesia, the molecular mechanisms underlying the long-lasting synaptic and cognitive impairments induced by transient exposure to anesthetics remain elusive. In the setting cancer and aging, mitochondrial injury and neuroinflammation have been associated with the increased production of free radicals and reactive oxygen species that resulted in epigenetic modifications of DNA methylation and histone acetylation.[12,13] Therefore, free radicals and reactive oxygen species could provide a link between the previously reported pathological phenomena[4,6,11] and the epigenetic modulation of brain-derived neurotrophic factor (BDNF) induced by neonatal anesthesia.

In the developing brain, synapse connectivity and dendritic spines undergo significant remodeling.[14] Exposure to anesthetics in the developing brain adversely affects the cerebral dendritic spine architectural plasticity in adulthood.[15] Among the biomolecules critically involved in the synaptogenesis and memory process, BDNF promotes the local translation of dendritic mRNAs of synaptic proteins[16] and plays a significant role in neuronal development, synaptic plasticity, and learning.[17,18] Although impaired BDNF signaling has been recently proposed to underlie anesthetic-induced neurotoxicity,[19] the molecular mechanisms that trigger BDNF inhibition and loss of synaptic plasticity remain unknown. Meanwhile, evidence suggests that potential epigenetic modification mediated by transcriptional factors (histone deacetylase 2 [HDAC2], methyl-cytosine-phosphate-guanine (CpG)–binding protein 2 [MeCP2], and DNA methyltransferase 1 [DNMT1]) can substantially induce chromatin remodeling in the promoter regions of the *Bdnf* gene, which can subsequently modulate the hippocampal synaptogenesis and cognitive function, in several neurological disorders.[20,21]

Hence, we explored the epigenetic mechanism underlying the persistent alteration of hippocampal BDNF signaling in rats with neonatal anesthesia. The current rat experiments were conducted during time periods that approximate the neonatal-to-adulthood developmental stages in humans.[22]

Exposure to environmental enrichment (EE), characterized with enhanced interactive social and physical activity, has been demonstrated to substantially modulate the synaptogenesis and synaptic plasticity in the central neurons,[23–25] thus improving memory function in rodents.[23,26,27] Exposure to an enriched environment mitigated sevoflurane-induced impairment in short-term memory in adult rodents with neonatal anesthesia.[11,28] Furthermore, studies in humans have shown that there is a relation among parental care, physical activity, and social interaction in stimulating memory development.[29] Similar effects of EE have also been noted in nonhuman primates.[30] This study further elucidates the molecular mechanism underlying the potential therapeutic effect of EE on memory deficiency induced by neonatal anesthesia. We hypothesized that exposure of the developing brain to anesthesia triggers epigenetic modification, involving the enhanced interaction among transcription factors (HDAC2, MeCP2, and DNMT1), in *Bdnf* promoter region(s) that inhibit BDNF expression, resulting in insufficient drive for local translation of mRNAs of synaptic proteins. We further hypothesized that noninvasive EE will attenuate anesthesia-induced epigenetic inhibition of BDNF signaling and memory loss in rodent models.

Materials and Methods

Animals and Neonatal Anesthesia

All animal procedures were approved by the Animal Care and Use Committee of Cleveland Clinic. To avoid the confounding effect of sex difference on the effects of neonatal anesthesia,[31,32] only male Sprague-Dawley rats were used for all experiments. Seven days after birth (P7), neonatal animals were randomly assigned (one half of each litter) to receive either isoflurane anesthesia (2.5% induction, 1.5% maintenance in 100% oxygen) for 6 h or sham anesthesia (control; the second one half of each litter). A P7 rat is at the same neurodevelopmental stage as a 0- to 6-month-old human.[22] An in-line anesthetic humidifier was used during anesthesia, and subcutaneous fluids (0.25 ml of 0.9% sterile NaCl intraperitoneally) were administered. This model may not be necessarily relevant in the context of routine care of human neonates as the anesthetized pups were not mechanically ventilated and normocapnia was not controlled during anesthesia. P7 sham anesthesia pups were removed from their home cage and placed into a prewarmed induction chamber. The lid was left open, the anesthesia vaporizer was turned off, and the animals breathed room air. After 6 h, pups who received anesthesia (when they are awake and vocalizing) and sham anesthesia pups were placed back into their home cage with their dams. They were initially observed closely to ensure that the dam accepted them and then at least once more before the end of the day and then at least daily for the next 3 days. The results of several studies have shown that this protocol causes substantial neurodegenerative damage to developing neurons.[6,33–36] Vital signs were monitored, and rectal temperature was maintained around 37° ± 0.5°C by a means of a heating pad. Behavioral, molecular, and electrophysiological studies were performed on rats on P65. The animals were randomly assigned to different groups with specific treatment, and another party blinded the experimenter to the individual groups.

Environmental Enrichment

On P21, pups were weaned, and animals were randomly assigned to EE or a standard cage environment (no EE). The EE animals were placed into groups of six animals living in large 60 × 30 × 60-cm cages, which were filled with toys and running wheels; other enrichment objects were regularly changed and replaced daily for 6 weeks.[27] This enriched cage environment provided greater opportunities for physical exercise, social interaction, and exposure to novel learning tasks compared with non-EE animals, which were weaned at P21

to two animals per cage in a standard size 20 × 40 × 30-cm clear plastic cage with no EE. In all groups, the food and water were provided *ad libitum*. All animals were maintained on a light:dark (12 h:12 h) schedule, with lights on at 7:00 AM.

Microinjection into the Hippocampal CA1 Area

For local administration of BDNF or a recombinant extracellular domain of human tropomyosin–related kinase receptor type B fused to the Fc region of human IgG (TrkB-Fc chimera), bilateral cannulae were implanted in a subset of animals at P53, as described by our group.[37] The rats were anesthetized with sodium pentobarbital (45 mg/kg intraperitoneally) and restrained in a stereotaxic apparatus. A 26-gauge double-guide cannula was inserted into the brain, aimed at the hippocampal CA1 area (anteroposterior, −3.5 mm; mediolateral, ±2.0 mm; dorsoventral, −3.0 mm).[38] The guide cannula was then cemented in place to the skull and securely capped. The rat was allowed to recover for at least 5 d before subsequent treatment. BDNF (50 ng per side for 7 days), Trkb-Fc chimera (2 μg per side for 7 days), or equivalent volume of saline (3 μl per side for 7 days) was delivered into the hippocampal CA1 area through a 33-gauge double injector with a rate of 0.5 μl/min. The injection sites for the hippocampal CA1 were histologically verified afterward by injecting the same volume of ink.[39,40]

Morris Water Maze Test

Morris water maze test (n = 10 in each group) was employed to determine the memory function of the rats as described by our group.[37,40] The water maze test was performed in a circular tank (diameter, 1.8 m) filled with opaque water. A platform (15 cm in diameter) was submerged below the water's surface in the center of the target quadrant. The platform was camouflaged by placing opacifying material (tempera paint) in the water. The swimming path of the rat was recorded by a video camera and analyzed by EthoVision XT software (Noldus Information Technology, USA). For each training session, the rats were placed into the maze consecutively from four random points of the tank and were allowed to search for the platform for 120 s. If the rat did not find the platform within 120 s, it was gently guided to it. Rats were allowed to remain on the platform for 20 s. The latency for each trial was recorded for analysis. During the probe trial (memory retrieval on day 6), the platform was removed from the tank, and the rats were allowed to swim in the maze for 60 s.

Chromatin Immunoprecipitation

The chromatin immunoprecipitation (ChIP) assay was performed as described by our group[37,41] with minor modifications. The hippocampal CA1 tissues were collected and crosslinked with 2% formaldehyde for 5 min, and chromatin was solubilized and sonicated with a sonic Dismembrator 250 (Fisher Scientific, USA) on ice six times for 30 s each followed by 1 min cooling on ice to produce fragments of approximately 200 to 500 base pair (bp). The polyclonal antibody against histone H3 acetylated at the lysine residues of *N* terminus, anti-HDAC2 antibody, anti-MeCP2 antibody, or anti-DNMT1 antibody (1:100, Millipore, USA) were added to each sample and incubated overnight at 4°C with gentle mixing. Mouse or rabbit IgG was used as the negative control, and monoclonal anti-RNA polymerase II antibody (1:100, Millipore) was used as a positive control. Immunocomplexes were recovered by salmon sperm DNA–protein A agarose beads and sequentially extensively washed. The crosslinking between histones and DNA was reversed, and DNA fragments were purified with phenol–chloroform extraction followed by acid ethanol precipitation.

Real-time polymerase chain reaction (PCR) was performed to amplify approximately 200 bp fragments within the transcriptional control regions. Primers sets were as follows: *Bdnf* exon IV: 5′-ATGCAATGCCCTGGAACGGAA-3′, 5′-TAGTGGAAATTGCATGGCGGAGGT-3′ and *Gapdh*: 5′-AGACAGCCGCATCTTCTTGT-3′, 5′-CGTCCTCTACCATCCTCTGC-3′. Amplifications were run in triplicate. $\Delta Ct_{[\text{normalized ChIP}]} = (Ct_{[\text{ChIP}]} - [Ct_{[\text{Input}]} - \text{Log}_2\{\text{input dilution factor}\}])$; and ChIP:input ratio was calculated as $2^{(-\Delta Ct\ [\text{normalized ChIP}])}$.

Methylated DNA Immunoprecipitation

Methylated DNA immunoprecipitation was performed as described by our group with minor modification.[42] Hippocampal CA1 tissue was homogenized, and genomic DNA was sonicated on ice six times for 30 s each. A polyclonal antibody against 5-methylcytosine (1:100, Millipore) was added to each sample end incubated overnight at 4°C with gentle mixing. Immunocomplexes were recovered by salmon sperm DNA–protein A agarose beads and successively washed, and DNA fragments were purified with phenol–chloroform extraction followed by acid ethanol precipitation. Immunoprecipitated DNA was subjected to quantitative real-time PCR using primers specific for about 250-bp segments corresponding to CpG sites within *Bdnf* exon IV promoter region. Primers sets were as follows: *Bdnf* exon IV: 5′-ATGCAATGCCCTGGAACGGAA-3′, 5′-TAGTGGAAATTGCATGGCGGAGGT-3′ and *Gapdh*: 5′-AGACAGCCGCATCTTCTTGT-3′, 5′-CTGCGGGAGAAGAAAGTCAG-3′. Amplifications were run in triplicate, and the PCR data were analyzed as described in the last paragraph of the Chromatin Immunoprecipitation section.

Quantitative Reverse Transcription Polymerase Chain Reaction for mRNA Analysis

RNA was isolated from the hippocampal CA1 tissue using a single-step RNA isolation protocol and quantified.[37,43] Reverse transcription and real-time reverse transcription PCRs were performed in triplicate with the following primers: *Bdnf* exon I: 5′-CTCAAAGGGAAACGTGTCTCT-3′, 5′-TCACGTGCTCAAAAGTGTCAG-3′; *Bdnf* exon II: 5′-CTAGCCACCGGGGTGGTGTAA-3′,

5′-TCACGTGCTCAAAAGTGTCAG-3′; *Bdnf* exon IV: 5′-TGCGAGTATTACCTCCGCCAT-3′, 5′-TCACGTGCTCAAAAGTGTCAG-3′; *Bdnf* exon VI: 5′-TTGGGGCAGACGAGAAAGCGC-3′, 5′-TCACGTGCTCAAAAGTGTCAG-3′; *Gapdh*: 5′-AGACAGCCGCATCTTCTTGT-3′, 5′-CTTGCCGTGGGTAGAGTCAT-3′. Fold change was calculated using the ΔΔCT method. The entire protocol was repeated in triplicate, and the mean and SEM were calculated.

Synaptosomal and Total Protein Preparation and Immunoblotting

The protocol for preparing synaptosomes was generally based on our previous report.[44] Hippocampal CA1 tissues from the rats in the appropriate groups were gently homogenized in ice-cold 0.32 M sucrose buffer at pH 7.4 and then centrifuged for 10 min at 1,000*g* (4°C). The supernatant was collected and centrifuged for 20 min at 10,000*g* (4°C). The synaptosomal pellet was then resuspended in the lysis buffer (0.1% Triton X-100, 150 mM NaCl, 25 mM KCl, 10 mM Tris–HCl, pH 7.4, with protease inhibitors) at 4°C for 10 min. For total protein preparations, the hippocampal tissues from the rats were homogenized on ice for 10 min in the lysis buffer containing 50 mM Tris–HCl, 150 mM NaCl, 0.02 mM NaN_2, 100 μg/ml phenylmethyl sulfonyl fluoride, 1 μg/ml aprotinin, and 1% Triton X-100. The lysates were centrifuged at 14,000 rpm for 10 min at 4°C, and the supernatant was used for sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis. Protein concentrations were determined by using the Bio-Rad Laboratories (USA) protein assay kit.

The protocol for immunoblotting was performed with hippocampal total protein and synaptosomal preparations.[37] The protein preparations were subjected to 7.5 to 15% SDS-polyacrylamide gel electrophoresis followed by immunoblotting. The blots were incubated overnight at 4°C with the primary antibodies as follows: polyclonal anti-BDNF antibody (1:300; Santa Cruz Biotechnology, USA), monoclonal anti-HDAC2 antibody (1:1,000; Cell Signaling, USA), monoclonal anti-DNMT1 antibody (1:1,000; Cell Signaling), rabbit polyclonal antibody against MeCP2 antibody (1:1,000; Millipore), phosphorylated eukaryotic translation initiation factor 4 (eIF4; 1:1,000; Cell Signaling), phosphorylated 70-kDa ribosomal protein S6 kinase (p70-S6 kinase; 1:1,000; Cell Signaling), p-S6 (1:1,000; Cell Signaling), GluR1 (1:1,000; Cell Signaling), SV2 (1:1,000; Cell Signaling) or CaMKIIα (1:1,000; Cell Signaling), or monoclonal anti-β-actin antibody (1:1,000; Santa Cruz Biotechnology). The membranes were washed extensively and then incubated with horseradish peroxidase–conjugated anti-mouse and anti-rabbit IgG antibody (1:15,000; Jackson ImmunoResearch Laboratories, USA). The immunoreactivity was detected using enhanced chemiluminescence (ECL Advance Kit, Amersham Biosciences, USA). The intensity of the bands was captured digitally and analyzed quantitatively with ImageJ software (NIH, USA). The immunoreactivity of all proteins was normalized to that of β-actin.

Coimmunoprecipitation and Immunoblotting

Coimmunoprecipitation and immunoblotting were performed.[37,38] Hippocampal CA1 tissues were homogenized in the lysis buffer (0.1% Triton X-100, 150 mM NaCl, 2.5 mM KCl, and 10 mM Tris–HCl, pH 7.4) with protease inhibitors and phosphatase inhibitors. Cleared lysate was incubated overnight at 4°C with rabbit polyclonal anti-HDAC2 or ant-DNMT1 antibody (1:100, Cell signaling) and precipitated for 5 h by protein A agarose beads (Sigma Aldrich, USA). Precipitates were washed extensively, and the proteins were separated on a 7.5% SDS-polyacrylamide gel and blotted to nitrocellulose membrane. The blots were incubated overnight at 4°C with monoclonal antibody of anti-MeCP2 (1:200; Santa Cruz Biotechnology). The membrane was washed with Tris-buffered saline and incubated with horseradish peroxidase–conjugated anti-mouse IgG antibody (1:10,000, GE Healthcare, USA) with enhanced chemiluminescence detection thereafter. The total amount of immunoprecipitated HDAC2 or DNMT1 was detected by incubating the stripped membranes with monoclonal anti-HDAC2 or anti-DNMT1 antibody (1:100; Santa Cruz Biotechnology). The intensity of the bands was captured digitally and analyzed quantitatively with ImageJ software.

Golgi Impregnation

Golgi impregnation was performed with the FD rapid GolgiStain kit (FD NeuroTechnologies, Inc., USA).[37,45] Golgi–Cox-stained brains were cut to cross-sections of 100-μm thickness with a Vibratome (Leica Biosystems, Germany) and digitized and analyzed using Image-Pro Plus software (Media Cybernetics, USA). For each experimental group, a minimum of 10 cells per slice (5 animals) were analyzed. The number of apical and basal spines on hippocampal CA1 pyramidal neurons was blindly counted.

Transmission Electron Microscopy

Rat brains (four or five per group) were perfused in 0.2 M sodium cacodylate buffer (pH 7.4) containing 2.5% glutaraldehyde and 4% formaldehyde. After washing three times in the same buffer, hippocampal CA1 tissues were postfixed with 1% aqueous osmium tetroxide for 1 h at room temperature (22° to 24°C). The tissues were washed in sodium cacodylate buffer followed by maleate buffer (pH 5.1) and then dehydrated with ascending grades of ethanol and propylene oxide. Samples were embedded in LX-112 medium, polymerized at 70°C for 48 h, trimmed, sectioned at 70 to 90 nm, poststained in 50% saturated uranyl acetate and 0.2% lead citrate, and examined with a transmission electron microscope (Philips CM120, The Netherlands).[46] Sections were analyzed in a blinded fashion.

Electron Microscopy Quantification

At least 21 single-section transmission electron micrographs were randomly taken from dense neuropil of the hippocampal CA1 area.[47] The area of each image taken at 23,000× was approximately 27.7 μm^2. Synapses were identified based on the generally accepted criteria.[47]

Hippocampal Slice Preparation and Whole Cell Recordings

Brain slices containing hippocampal CA1 area were prepared.[37,38,48] The brain was quickly removed and cut on a Vibratome in cold physiological saline to obtain coronal slices (300-μm thick) containing the hippocampus. Whole cell voltage clamp recordings from the CA1 area were taken using an Axopatch 200B amplifier (Molecular Devices, USA) with 2- to 4-MΩ glass electrodes containing the internal solution (millimolar): 125 cesium methanesulfonate; 5 NaCl; 1 $MgCl_2$; 0.5 EGTA; 2 Mg-ATP; 0.1 Na_3GTP; 10 HEPES; pH 7.3; and 290 to 300 mOsmol. A seal resistance of more than or equal to 2 GΩ and an access resistance of 15 to 20 MΩ were considered acceptable. The series resistance was optimally compensated by more than or equal to 70% and constantly monitored throughout the experiments. Schaffer collateral–commissural fibers were stimulated by ultrathin concentric bipolar electrodes (FHC Inc., USA), and the excitatory postsynaptic currents (EPSCs) were recorded in the CA1 area in the presence of bicuculline (30 μM). The evoked EPSCs were filtered at 2 kHz, digitized at 10 kHz, and acquired and analyzed using Axograph X software (Australia). The amplitude of the EPSCs was monitored for a baseline period of at least 15 min. If synaptic transmission was stable (less than 15% change in EPSC amplitude over 15 min), LTP was induced by a single high-frequency electric stimuli train (100 Hz for 1 s).[49] All electrophysiological experiments were performed at room temperature.

Compounds

D-2-amino-5-phosphonopentanoate, 6-cyano-2,3-dihydroxy-7-nitroquinoxaline, bicuculline, and other chemicals were purchased from Sigma Aldrich (USA) or Tocris (USA). Recombinant human TrkB-Fc chimera was purchased from R&D Systems, Inc. (USA).

Data Analysis

No statistical methods were used to predetermine sample sizes, but our sample sizes are similar to those reported in previous publications.[37] The sample size was unintentionally not even in some experiments. There were no missing data, and no data were excluded to have uniformity in the sample size. Normality was tested using Shapiro and Wilk test for normality. Electrophysiological data were analyzed with repeated measures ANOVA. Behavioral data were compared using a general mixed model analysis of variance. The data from histological study, western blot, real-time PCR and other studies were analyzed using Student's *t* test, one-way ANOVA test, or Kruskal-Wallis test (for nonnormally distributed data). *Post hoc* analyses were performed using Tukey studentized range method or Dunn multiple comparisons test as appropriate. All statistical analyses were performed with BMDP statistical software (Statistical Solutions, USA). All data were expressed as means ± SD. For all tests, a two-tailed $P < 0.05$ was considered statistically significant.

Results

EE Reverses Neonatal Anesthesia-induced Memory Deficiency and Hippocampal Synaptic Loss

To characterize the cognitive effects of neonatal anesthesia and the potential therapeutic effect of EE, we performed the Morris water maze test in adult rats at P65 that previously received isoflurane anesthesia at P7. Consistent with previous reports,[4] the rats that previously received isoflurane anesthesia exhibited memory deficiency as shown by extended escape latencies and less time spent in the target quadrant (during the probe trial) in the Morris water maze test (fig. 1, A and B). Exposure to the enriched environment (24 h per day for 6 weeks starting at P21) substantially restored cognitive function as evidenced by decreased escape latency and increased time spent in the target quadrant in the rats with neonatal anesthesia (fig. 1, A and B). These findings indicate that the enriched environment substantially ameliorated the memory deficiency in P65 rats that had previously received anesthetics.

Having confirmed anesthesia-induced cognitive dysfunction, we recorded electric stimuli–induced LTP in the hippocampal CA1 neurons to evaluate hippocampal synaptic plasticity and the potential effect of EE. P65 rats with neonatal anesthesia displayed significantly impaired glutamatergic LTP in the hippocampal CA1 neurons (fig. 1C), indicating an impaired synaptic plasticity. We also found that exposure to the enriched environment appreciably recovered the electric stimuli–induced LTP in the hippocampal CA1 neurons in P65 rats with neonatal anesthesia, whereas the effect was marginal in the control rats (fig. 1C). Because the synapse is assumed to be the cellular basis for learning and memory[50] and dendrites are essential integration points of synaptic information for neurons,[51] we assessed the change in dendritic spine numbers and synaptic ultrastructure in the hippocampal CA1 in adult rats that had neonatal exposure to anesthesia and the potential effect of EE. Indeed, hippocampal glutamatergic synaptic densities and dendritic spine numbers were reduced in the hippocampal CA1 neurons of the rats exposed to neonatal anesthesia (fig. 1, D and E); these effects were restored by EE. Taken together, these results suggest that EE reinstated learning behavior, remarkably recovered the morphological deconstruction of hippocampal synapses, and restored hippocampal synaptic plasticity induced by neonatal anesthesia.

EE Reverses Neonatal Anesthesia-induced Reduction of Hippocampal BDNF

Because BDNF plays a critical role in modulating the translation of synaptic protein,[16] synaptogenesis, synaptic plasticity,

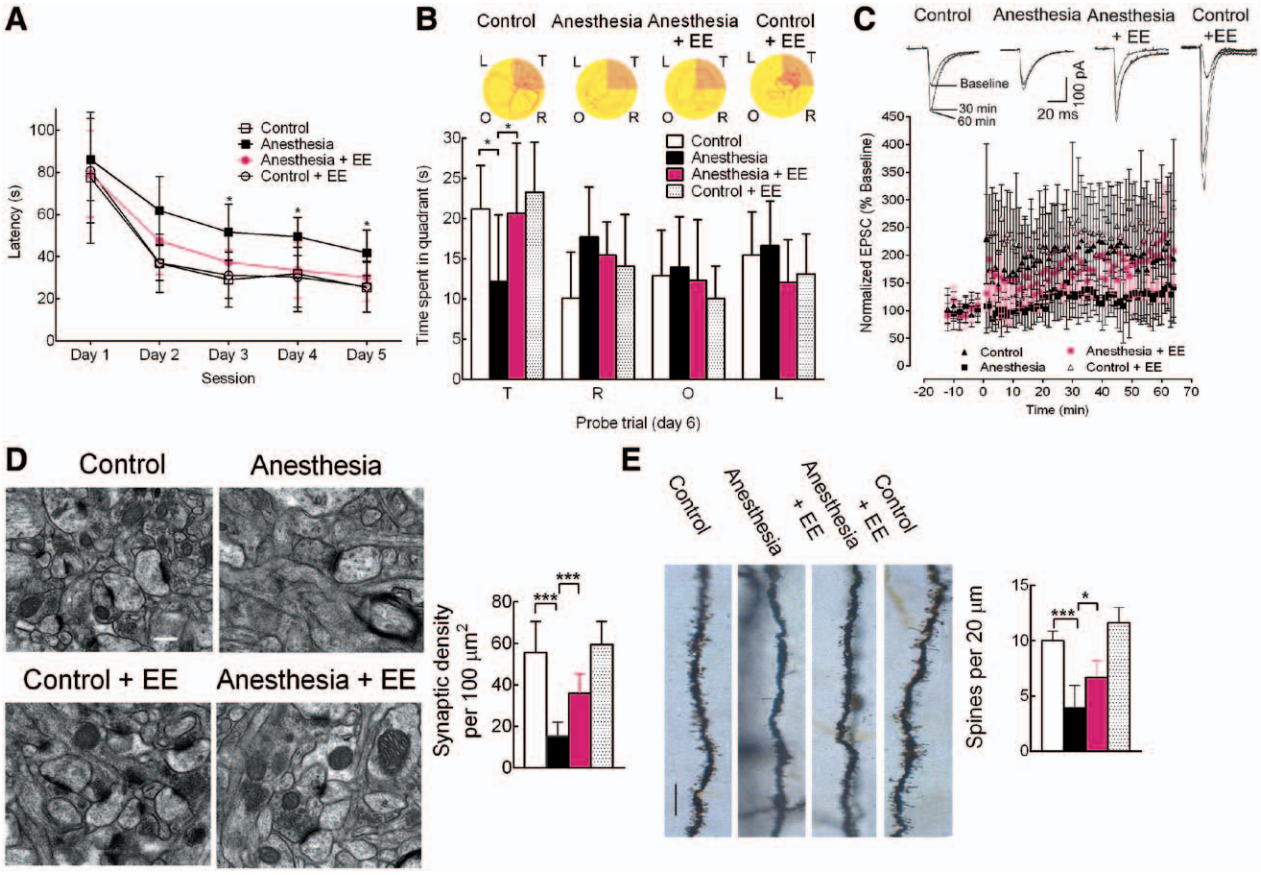

Fig. 1. Environmental enrichment (EE) significantly improved performance in the Morris water maze test and recovered the hippocampal synaptic plasticity in the rats with neonatal anesthesia. (*A*, *B*) Significantly extended escape latency (n = 10 rats in each group, effect of group [$F_{3, 36}$ = 15.9, P < 0.0001], effect of time [$F_{4, 36}$ = 62.9, P < 0.0001], interaction between group and time [P = 0.9]) and less time spent in the target quadrant (n = 10 rats in each group, effect of group $F_{3, 36}$ = 4.5, P = 0.0086) in P65 rats that previously received isoflurane anesthesia at P7 but not in rats exposed to EE after neonatal anesthesia. (*B*) Representative path tracings in each quadrant during the probe trial on day 6 (T, target quadrant; R, right quadrant; O, opposite quadrant; L, left quadrant). We observed significantly impaired high-frequency stimulation-induced long-term potentiation of excitatory postsynaptic current (EPSC; *C*, n = 11, 8, 8, and 8 neurons, from four to six rats in each group, effect of group [($F_{3, 31}$ = 6.3, P = 0.0018], effect of time [P < 0.0001], interaction between group and time [P = 0.3]) in hippocampal CA1 neurons in the rats with neonatal anesthesia, which was recovered by the exposure to an enriched environment. Neonatal exposure to anesthetics also significantly reduced hippocampal synaptic density (*D*, n = 5 rats in each group, $F_{3, 16}$ = 72.9, P < 0.0001, scale bar = 0.25 μm) and dendritic spine numbers (*E*, n = 5 rats in each group, $F_{3, 16}$ = 25.7, P < 0.0001, scale bar = 10 μm) in the adult rats, which were restored by the enriched environment. Total synapse density was calculated as the number of synapses per 100 μm^2 of neuropil. *P < 0.05; ***P < 0.001. Data represent mean ± SD.

and the learning process,[17,18] we then examined the effects of EE on the expression of BDNF in the hippocampal CA1 of the adult rats that received anesthetics at P7. As shown in figure 2A, the reduction of BDNF expression in the hippocampal CA1 in rats with neonatal anesthesia was substantially recovered after exposure to the enriched environment.

To further characterize the changes of BDNF in the adult rats with neonatal anesthesia, we examined *Bdnf* mRNA levels in the hippocampal CA1 area. The *Bdnf* gene has a complicated structure in that it consists of nine 5′ noncoding exons, each with independent promoter regions and a 3′ coding exon.[52] To this end, we performed retroscribed real-time PCR to screen the potential change of isoform-specific transcription initiation in the *Bdnf* gene locus. We observed a marked reduction in the mRNA of *Bdnf* exon IV but not exon I, II, and VI in the hippocampal CA1 in the rats with neonatal anesthesia (fig. 2, B and C). This reduction of *Bdnf* exon IV was significantly recovered by exposure to the enriched environment (fig. 2C). Taken together, these data indicate that BDNF plays a role in anesthetic-induced memory deficiency and in the therapeutic effect of EE.

EE Reverses Neonatal Anesthesia-induced Epigenetic Suppression of Bdnf

Epigenetic modification of the promoter region of *Bdnf* exons appears to substantively modulate the expression of BDNF, thereby regulating synaptogenesis, synaptic plasticity, and memory.[42] MeCP2 is a transcriptional repressor

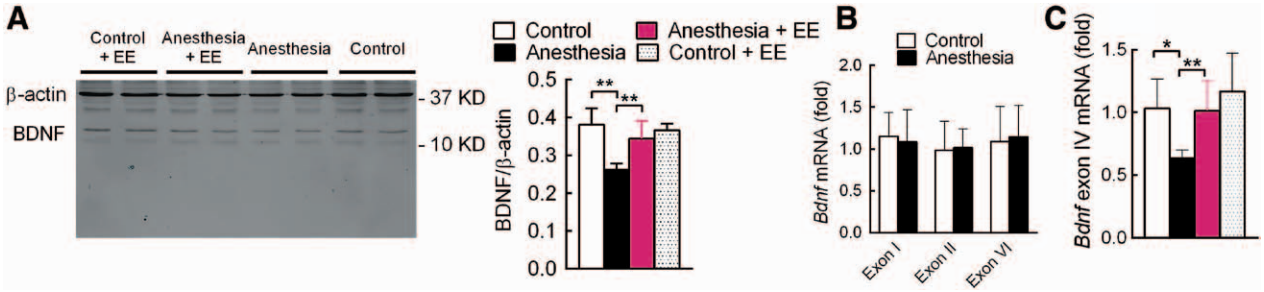

Fig. 2. Neonatal exposure to anesthetics significantly decreased the expression of brain-derived neurotrophic factor (BDNF; *A*, n = 6, 6, 7, and 7 rats in each group, $F_{3, 22} = 14.3$, $P < 0.001$) and *Bdnf* exon IV mRNA (*B* and *C*, n = 6 rats in each group, $F_{3, 20} = 6.00$, $P = 0.004$) in the hippocampal CA1 tissue in the adult rats, which was substantially ameliorated by environmental enrichment (EE). *$P < 0.05$; **$P < 0.01$. Data represent mean ± SD.

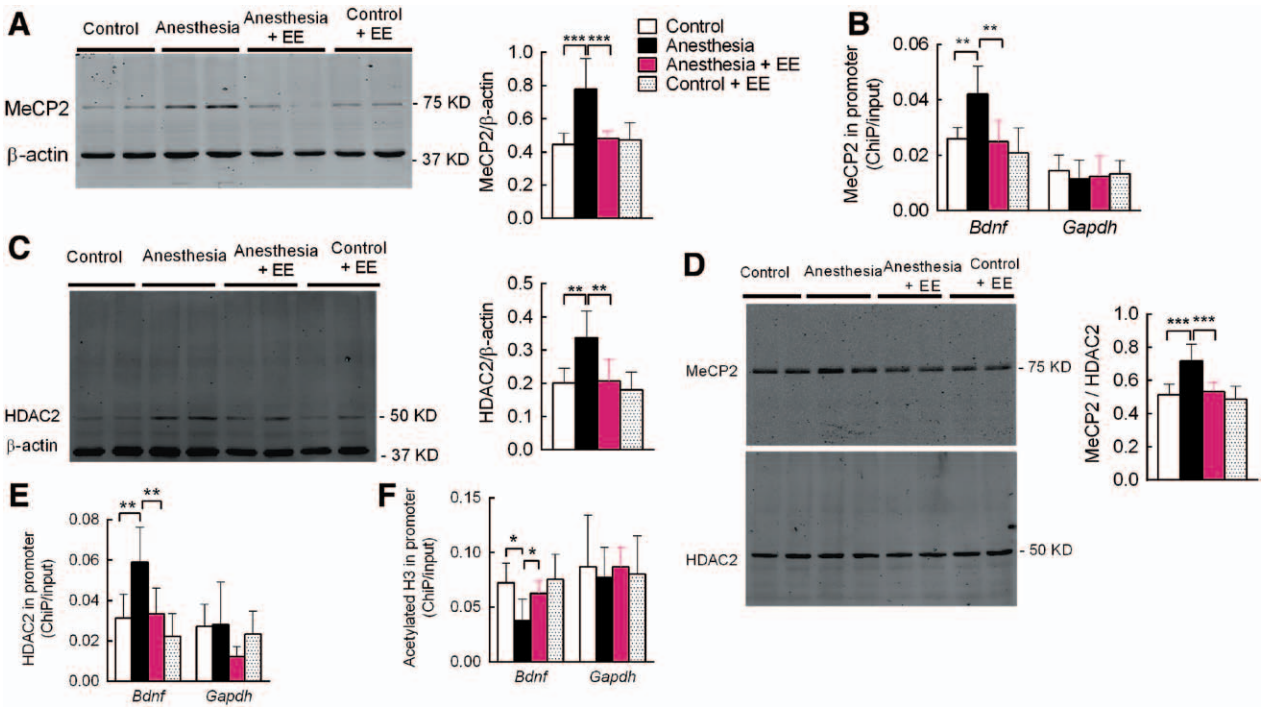

Fig. 3. Environmental enrichment (EE) recovered the histone H3 acetylation in the promoter region of *Bdnf* exon IV reduced by neonatal anesthesia. Significantly increased expression of methyl-cytosine-phosphate-guanine–binding protein 2 (MeCP2) (*A*, n = 6, 6, 7, and 7 rats in each group, $F_{3, 22} = 12.3$, $P < 0.0001$) and its occupancy in the promoter region of *Bdnf* exon IV (*B*, n = 6 rats in each group, $F_{3, 20} = 8.0$, $P = 0.0011$), but not *Gapdh*, was observed in the hippocampal CA1 in the rats with neonatal anesthesia, which was decreased by exposure to an enriched environment. Significantly increased expression of histone deacetylase 2 (HDAC2; *C*, n = 6, 6, 8, and 7 rats in each group, $F_{3, 23} = 8.0$, $P = 0.0008$) was observed in the hippocampal CA1 tissue in rats with neonatal anesthesia, which was decreased by EE. Also, an increased amount of MeCP2 in the immunocomplex pulled down by polyclonal HDAC2 antibody was observed in the hippocampal CA1 in the rats with neonatal anesthesia (*D*, n = 6, 6, 7, and 8 rats in each group, $F_{3, 23} = 12.5$, $P < 0.0001$), which was decreased by EE. Further chromatin immunoprecipitation (ChIP) study also revealed an increased HDAC2 occupancy (*E*, n = 6 rats in each group, $F_{3, 20} = 8.3$, $P = 0.0009$) and decreased histone H3 acetylation in the promoter region of *Bdnf* exon IV (*F*, n = 6 rats in each group, $F_{3, 20} = 5.1$, $P = 0.0084$) in rats with neonatal anesthesia, which was restored by the enriched environment. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. Data represent mean ± SD.

that binds to DNA sequences methylated at cytosine in the dinucleotide 5′ CpG,[53] and it appears to be involved in the maintenance of synaptic plasticity and cognitive functions in the mammal brain.[53,54] The functional adaptation of transcriptional repressor MeCP2 appears to regulate the expression of BDNF in the central nervous system.[55] In this study, significantly increased expression of MeCP2 was observed in the hippocampal CA1 area in the adult rats with neonatal anesthesia (fig. 3A). Further ChIP assays revealed increased occupancy of MeCP2 in the promoter region of *Bdnf* exon IV (fig. 3B). We also noted that exposure to the EE attenuated the MeCP2 expression (fig. 3A) and its occupancy in the promoter region of *Bdnf* exon IV (fig. 3B). Because MeCP2 acts as a general transcriptional repressor,

these results implied that the altered function of MeCP2 potentially contributed to the hippocampal BDNF reduction induced by neonatal anesthesia.

Histone deacetylases, including HDAC2, are recruited to the promoter region by MeCP2 resulting in histone deacetylation and subsequent repression of gene transcription.[56] Herein, we explored the functional adaptation of HDAC2 and its involvement in the BDNF reduction in the hippocampal CA1 area in adult rats with neonatal anesthesia. A significant increase in the expression of HDAC2 (fig. 3C) in the hippocampal CA1 tissue was also detected in the modeled rats (anesthetic-treated rats that were not exposed to the enriched environment). Further coimmunoprecipitation study revealed an increased amount of MeCP2 in the immunocomplex pulled down by polyclonal HDAC2 antibody, indicating an enhanced interaction between HDAC2 and MeCP2 in the hippocampal CA1 in the rats with neonatal anesthesia (fig. 3D). Further, ChIP study also revealed an increased HDAC2 occupancy (fig. 3E) and decreased histone H3 acetylation in the promoter region of *Bdnf* exon IV (fig. 3F). Note that exposure to the enriched environment significantly decreased the expression of HDAC2 (fig. 3C) and the immunocomplex consisting of HDAC2 and MeCP2 (fig. 3D) and recovered the occupancy of HDAC2 (fig. 3E) and acetylated histone H3 in the promoter region of *Bdnf* exon IV (fig. 3F). Considering that HDAC2 and acetylated histone H3 may regulate the transcription of the target gene, these findings suggest that altered histone H3 acetylation in the promoter region mediated by the HDAC2/MeCP2 interaction may contribute to the BDNF reduction induced by the neonatal anesthesia and that these effects were mitigated by EE.

Increased DNA methylation (5′-cytosine) in the promoter region is generally predictive of long-lasting suppression of the target gene. DNMT1 maintains a stable cytosine methylation state by partnering with MeCP2.[57] In this study, significantly increased expression of DNMT1 was observed in the hippocampal CA1 area of rats with neonatal exposure to anesthetics (fig. 4A). Coimmunoprecipitation study also revealed an increased amount of MeCP2 in the immunocomplex pulled down by polyclonal DNMT1 antibody, indicating an enhanced interaction between DNMT1 and MeCP2, in the hippocampal CA1 in the rats with neonatal anesthesia (fig. 4B). Furthermore, ChIP study revealed an increased DNMT1 occupancy (fig. 4C) and methylation of 5′-cytosine in the promoter region of *Bdnf* exon IV (fig. 4D). In contrast,

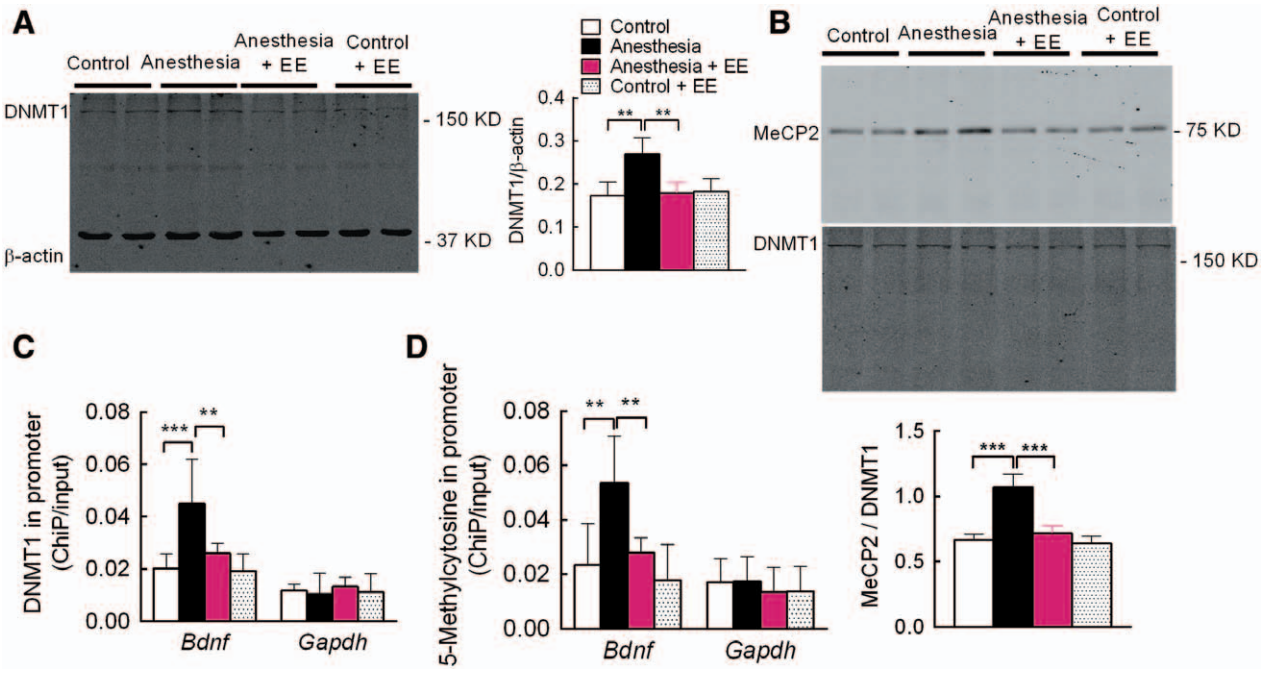

Fig. 4. Environmental enrichment (EE) reversed the increased 5′-cytosine methylation in the promoter region of *Bdnf* exon IV induced by neonatal anesthesia. Significantly increased hippocampal DNA methyltransferase 1 (DNMT1) was observed in the rats with neonatal anesthesia, which was significantly decreased by EE (*A*, n = 6, 6, 7, and 7 rats in each group, Kruskal-Wallis statistic = 13.5, $P < 0.0037$). Increased methyl-cytosine-phosphate-guanine–binding protein 2 (MeCP2) in the precipitated complex pulled down by polyclonal DNMT1 antibody in the hippocampal lysates was observed in the rats with neonatal anesthesia, which was reduced by EE (*B*, n = 6, 6, 7, and 7 rats in each group, $F_{3, 22} = 55.3$, $P < 0.0001$). Significantly increased occupancy of DNMT1 in the *Bdnf*, but not *Gapdh* promoter region, was observed in the rats with neonatal exposure to anesthesia, which was significantly decreased by EE (*C*, n = 6 rats in each group, $F_{3, 20} = 8.9$, $P = 0.0006$). Chromatin immunoprecipitation (ChIP) study with polyclonal antibody against 5′-methylcytosine showed a significant increase in cytosine methylation in the *Bdnf* exon IV, but not *Gapdh*, promoter region of the modeled rats, which was also reversed by EE (*D*, n = 6 rats in each group, $F_{3, 20} = 8.2$, $P = 0.0009$). $^{**}P < 0.01$; $^{***}P < 0.001$. Data represent mean ± SD.

exposure to the enriched environment significantly decreased the expression of DNMT1 (fig. 4A) and the immunocomplex consisting of HDAC2 and MeCP2 (fig. 4B) and recovered the occupancy of DNMT1 (fig. 4C) and methylation of 5′-cytosine in the promoter region of *Bdnf* exon IV (fig. 4D). Together, these findings show that altered cytosine methylation in the promoter region mediated by the DNMT1/MeCP2 internation may contribute to the BDNF reduction induced by the neonatal anesthesia and that EE has a potential therapeutic value in reversing these effects.

BDNF Reduction Contributes to the Synaptic Loss

To confirm the critical role of BDNF reduction in the neonatal anesthesia-induced memory deficiency, we investigated whether artificial suppression of endogenous BDNF signaling leads to hippocampal synaptic dysfunction and memory deficiency in naïve rats. To attain this strategy, BDNF-sequestering TrkB-Fc chimera (2 μg × 7 days) was microinjected into the hippocampal CA1 area in naïve rats. TrkB-Fc-microinjected rats exhibited extended escape latencies (fig. 5A) and spent less time in the target quadrant (fig. 5B) in the Morris water maze test, indicating an impaired cognitive function. A significantly impaired electric stimuli–induced LTP (fig. 5C), reduced glutamatergic synaptic density (fig. 5D), and decreased spine numbers (fig. 5E) were also observed in the hippocampal CA1 neurons in the rats injected with Trkb-Fc chimera. These results suggest that artificial suppression of BDNF signaling

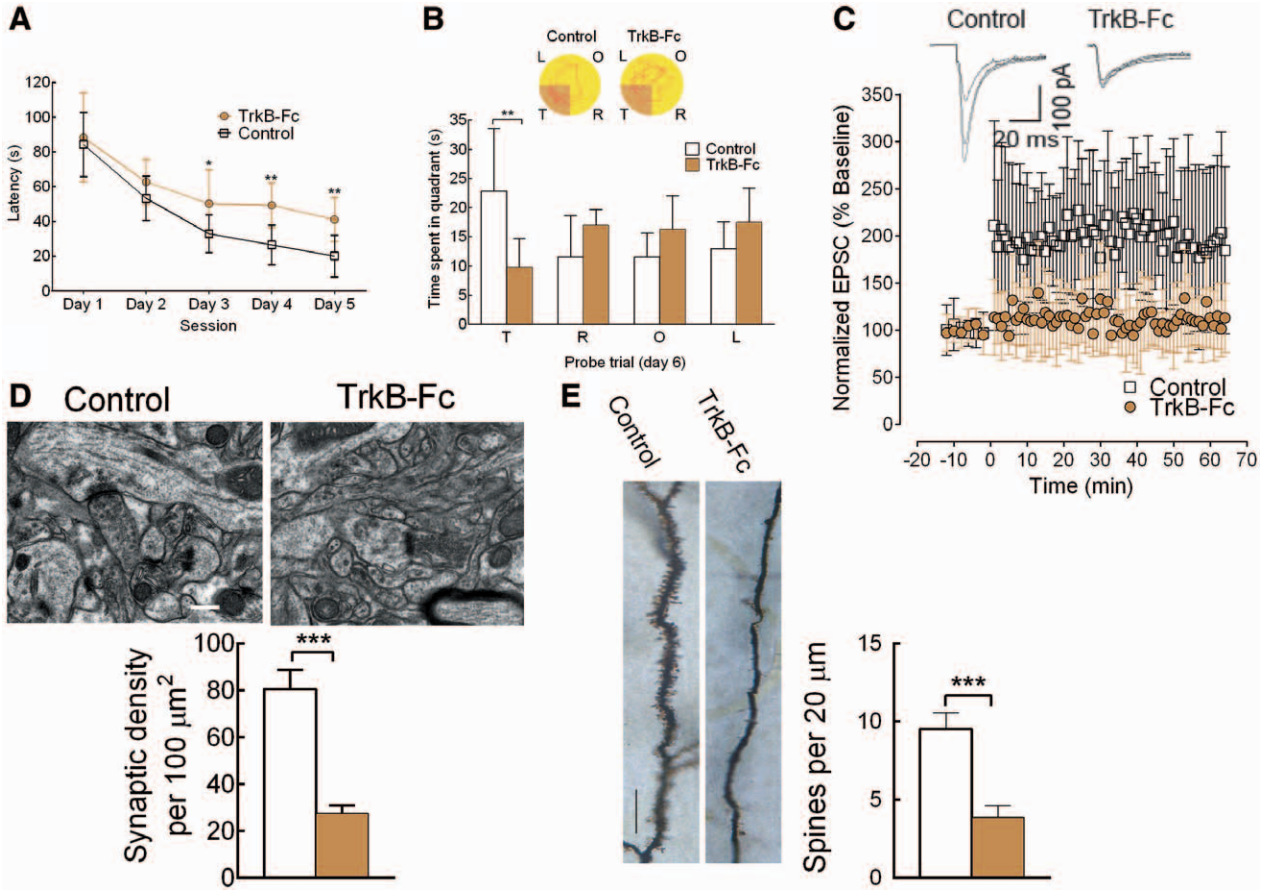

Fig. 5. Artificial suppression of hippocampal brain-derived neurotrophic factor signaling impaired hippocampal synaptic plasticity and memory. Microinjection of TrkB-Fc (tropomyosin receptor kinase B–Fc) chimera (a recombinant extracellular domain of human tropomyosin–related kinase receptor type B fused to the Fc region of human IgG; 2 μg × 7 days) into the hippocampal CA1 in naïve rats significantly extended the escape latencies (*A*, n = 10 rats in each group, effect of group [$F_{1, 18}$ = 13.6, *P* = 0.0017], effect of time [$F_{4, 18}$ = 48.6, *P* < 0.0001], interaction between group and time [*P* = 0.2]) and shortened the time spent in the target quadrant (*B*, n = 10 rats in each group, Kruskal-Wallis statistic = 7.41, *P* = 0.0065) in the Morris water maze test. (*B*) Representative path tracings in each quadrant during the probe trial on day 6 (T, target quadrant; R, right quadrant; O, opposite quadrant; L, left quadrant). Significantly impaired high-frequency stimulation-induced long-term potentiation of excitatory postsynaptic current (EPSC) was also observed in the hippocampal CA1 neurons in the naïve rats injected with Trkb-Fc chimera ([*C*, n = 10 neurons from five rats in each group, $F_{1, 18}$ = 17.42, *P* = 0.0006], effect of time [*P* = 0.94], interaction between group and time [*P* = 0.22]). Microinjection of Trkb-Fc chimera also significantly decreased hippocampal synaptic density (*D*, n = 5 rats in each group, t = 13.5, DF = 8, two-tailed *P* < 0.0001, scale bar = 0.25 μm) and dendritic spine numbers (*E*, n = 5 rats in each group, t = 10.0, DF = 8, two-tailed *P* < 0.0001, scale bar = 10 μm) in naive rats. Total synapse density was calculated as the number of synapses per 100 μm² of neuropil. **P* < 0.05; ***P* < 0.01; ****P* < 0.001. Data represent mean ± SD.

substantially mimicked the synaptic and cognitive dysfunction induced by neonatal anesthesia.

We then went on to test whether exogenous BDNF would attenuate synaptic and cognitive dysfunction induced by neonatal anesthesia. Local administration of exogenous BDNF (50 ng × 7days) into the hippocampal CA1 shortened the escape latencies (fig. 6A) and increased the time spent in the target quadrant (fig. 6B) in the Morris water maze test in the rats with neonatal anesthesia, indicating a recovery of cognitive function. Consistent with previous reports that BNDF is associated with increases in spine density and dimension,[51,58] we observed that BDNF treatment enhanced the electric stimuli–induced LTP in the hippocampal neurons (fig. 6C), increased glutamatergic synapses density (fig. 6D), and restored the hippocampal dendritic spine numbers (fig. 6D) in the modeled rats. Microinjection of exogenous BDNF had no obvious effect on the behavioral performance, synaptic plasticity, and synaptic ultrastructures in the control rats (fig. 6, A–E). These results suggested that supplementation of BDNF signaling recovered the hippocampal synaptic and cognitive function impaired by the neonatal anesthesia in the adult rodents.

BDNF Reduction Contributes to the Suppressed Translation of Synaptic mRNA

BDNF is known to stimulate the local translation of synaptic mRNA that regulates the synthesis of synaptic protein and synaptogenesis in the rodent brain.[16] Initiation of

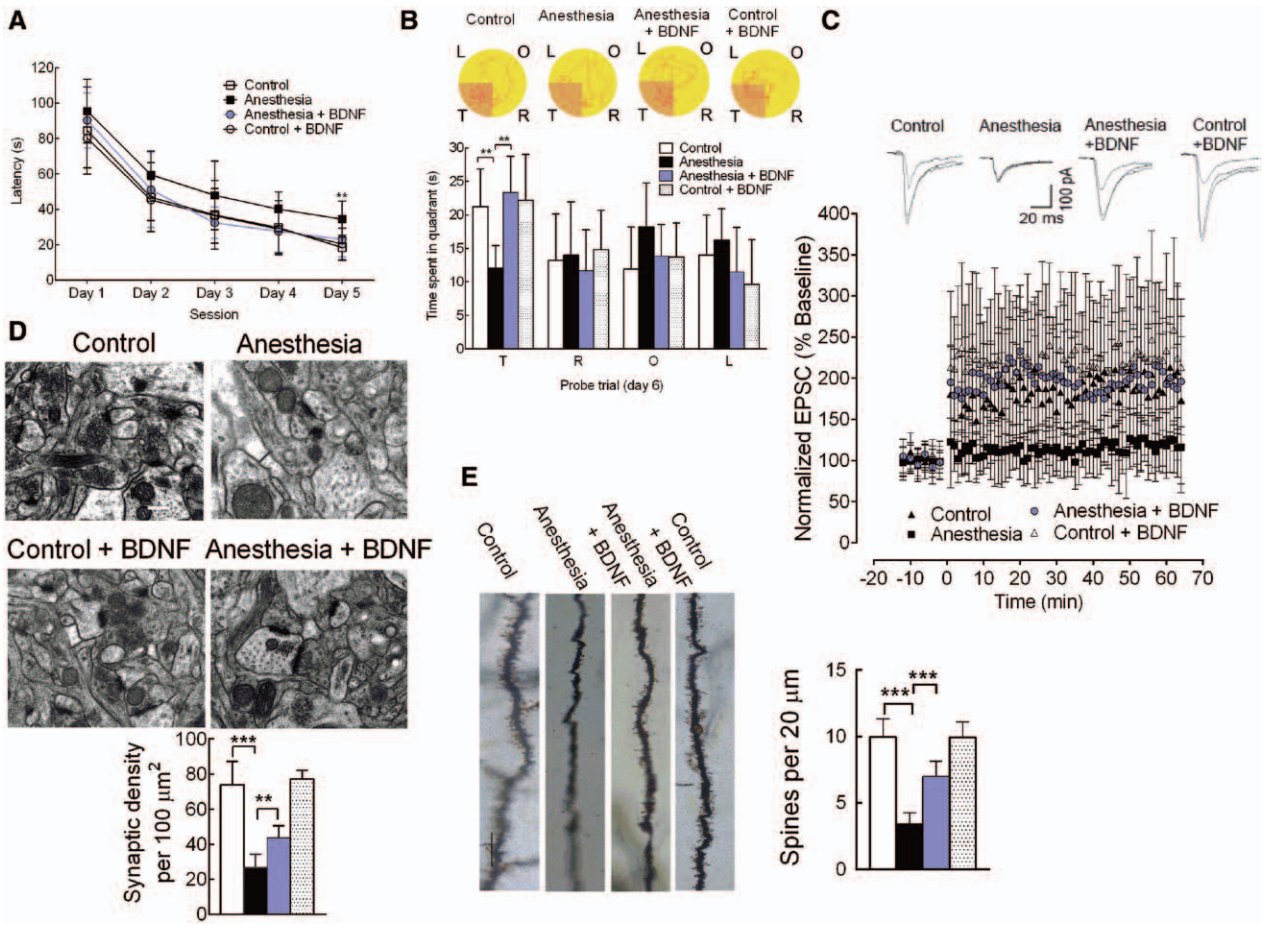

Fig. 6. Supplementation of brain-derived neurotrophic factor (BDNF; 50 ng × 7 days) substantially ameliorated the hippocampal synaptic dysfunction and memory deficiency induced by neonatal anesthesia. Microinjection of exogenous BDNF significantly shortened the escape latency (*A*, n = 10 rats in each group, effect of group [$F_{3, 36} = 8.03$, $P = 0.0003$], effect of time [$F_{4, 36} = 107$, $P < 0.0001$], interaction between group and time [$P = 0.98$]) and increased the time spent in the target quadrant (*B*, n = 10 rats in each group, $F_{3, 36} = 8.25$, $P = 0.0001$) in the Morris water maze test in the rats with neonatal anesthesia. (*C*) Representative path tracings in each quadrant during the probe trial on day 6 (T, target quadrant; R, right quadrant; O, opposite quadrant; L, left quadrant). Exogenous administration of BDNF also significantly enhanced the electric stimuli–induced long-term potentiation of excitatory postsynaptic current (EPSC) in the hippocampal neurons (*C*, n = 11, 11, 9, and 9 neurons from four to six rats, in each group [$F_{3, 37} = 6.75$, $P = 0.001$], effect of time [$P = 0.25$], interaction between group and time [$P = 0.95$]) and increased glutamatergic synapses (*D*, n = 5 rats in each group, $F_{3, 16} = 38.6$, $P < 0.0001$, *scale bar* = 0.25 μm) and hippocampal dendritic spine numbers (*E*, n = 5 rats in each group, $F_{3, 16} = 36.0$, $P < 0.0001$, *scale bar* = 10 μm) and in the hippocampal CA1 in rats with neonatal anesthesia. Total synapse density was calculated as the number of synapses per 100 μm² of neuropil. $^{**}P < 0.01$; $^{***}P < 0.001$. Data represent mean ± SD.

mRNA translation and protein synthesis–dependent synaptic plasticity and memory seem to be mediated in part by eIF4E, phosphorylated ribosomal protein S6, and p70-S6 kinase.[59–61] To evaluate the consequence of neonatal anesthesia-induced BDNF insufficiency, we then investigated the function of translation machinery and expression of several synaptic proteins in the synaptosomal preparations of hippocampal CA1 tissue in the rats with neonatal anesthesia. To this end, significantly decreased expression of phosphorylated eIF4, phosphorylated p70-S6 kinase, and phosphorylated S6 (fig. 7A) was observed in the synaptosomal preparation, indicating an inadequate function of synaptic translation machinery, in the hippocampal CA1 in the rats with neonatal anesthesia. We also found that exposure to the enriched environment, which increases the hippocampal expression of BDNF, significantly recovered the phosphorylation of translation machinery in the hippocampal synaptosomal preparations in the modeled rats; it did not induce obvious change in the control rats (fig. 7A). In addition, reduced expression of GluR1 (postsynaptic protein), SV2 (presynaptic protein), and CaMKIIα (a ubiquitous protein; fig. 7B) was detected in the hippocampal synaptosomal preparation in the modeled rats, indicating suppressed expression of synaptic proteins. We noted that after 6 weeks of EE, the levels of GluR1, SV2, and CaMKIIα were substantially increased in the modeled animals (fig. 7B), whereas no changes were seen in the control rats. These results demonstrated suppressed translational machinery and reduced synthesis of synaptic proteins in the hippocampal CA1, which potentially contributes to synaptic and cognitive dysfunction in the rats with neonatal anesthesia.

Next, we determined the involvement of BDNF signaling in the neonatal anesthesia-suppressed synaptic translational machinery. Microinjection of BDNF-sequestering TrkB-Fc chimera (2 μg × 7 days) significantly suppressed the expression of phosphorylated eIF4E, p70-S6 kinase, and S6 (fig. 8A) and decreased the expression of synaptic proteins GluR1, SV2, and CaMKIIα (fig. 8B) in the hippocampal synaptosomal preparation in the naïve rats. Taken together, these results further confirmed the critical role of BDNF reduction in hippocampal synaptic and cognitive dysfunction induced by neonatal anesthesia.

Discussion

BDNF has a critical role in promoting neuronal survival, synapse formation, and maturation in the developing brain as well as regulation of synaptic transmission in hippocampal and cortical neurons.[17] BDNF can induce a sustained increase of the synaptic efficacy at the Schaffer collateral-CA1 synapses,[62] and genetic disruption of *Bdnf* transcription results in impairment of LTP in hippocampal glutamatergic synapses, which can be restored by the introduction of exogenous BDNF.[63,64] Although the rat with cognitive deficiency induced by chronic cerebral ischemia exhibited a reduction of the hippocampal BDNF level,[65] increased central BDNF signaling was associated with the growth hormone–induced improvement of cognitive function in the rodent model of traumatic brain injury.[66] In contrast to a previous study noting an inconsistent change of BDNF in several brain regions in neonate rats exposed to propofol anesthesia,[19] this study revealed a sustained reduction of hippocampal BDNF in the adult rats with neonatal anesthesia. We also found that the introduction of exogenous BDNF was effective in restoring hippocampal synaptic plasticity and cognition in the modeled rats, whereas artificial suppression of hippocampal BDNF signaling in naïve rats with TrkB-Fc chimera emulated the hippocampal synaptic and cognitive impairments induced by neonatal anesthesia. These results support the

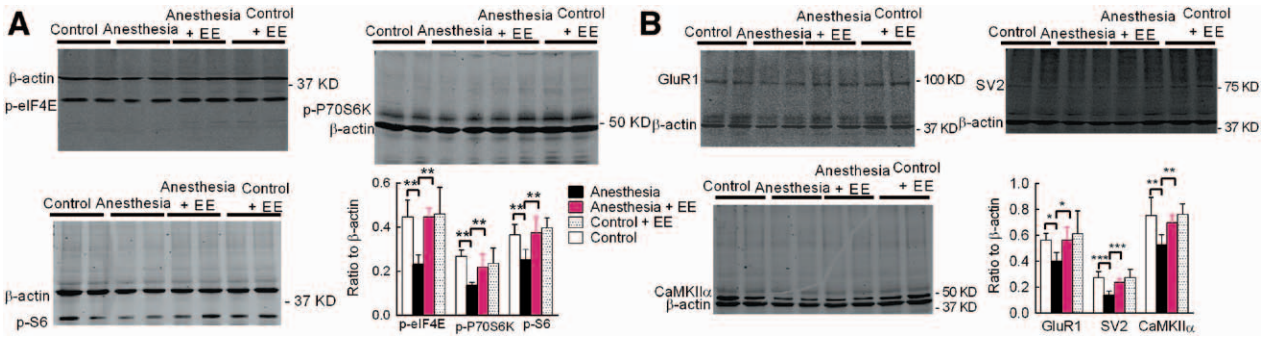

Fig. 7. Environmental enrichment (EE) significantly restored the synthesis of synaptic proteins in the hippocampal synaptosomal preparations in the rats with neonatal anesthesia. (*A*) Significantly decreased phosphorylation of eukaryotic translation initiation factor 4E (eIF4E; n = 6, 6, 7, and 7 rats in each group, $F_{3, 22} = 12.3$, $P < 0.001$), phosphorylated 70-kDa ribosomal protein S6 kinase (n = 6 rats in each group, $F_{3, 20} = 8.8$, $P = 0.001$), and S6 (n = 6 rats in each group, $F_{3, 20} = 8.6$, $P = 0.0007$) was observed in the hippocampal CA1 synaptosomal preparation in the rats with neonatal anesthesia, which was recovered by the enriched environment. (*B*) Significantly decreased phosphorylation of GluR1 (a postsynaptic protein; n = 6 rats in each group, $F_{3, 20} = 4.3$, $P = 0.0164$), SV2 (n = 6 rats in each group, $F_{3, 20} = 12.2$, $P < 0.0001$), and calcium/calmodulin-dependent protein kinase II α (CaMKIIα; n = 6 rats in each group, $F_{3, 20} = 8.1$, $P = 0.001$) was observed in the hippocampal CA1 synaptosomal preparation in the rats with neonatal anesthesia, which was recovered by the enriched environment. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. Data represent mean ± SD.

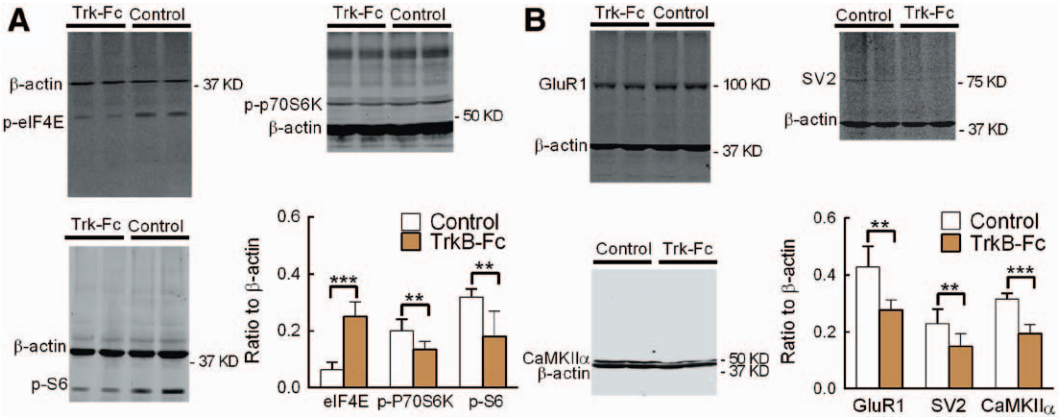

Fig. 8. Suppression of brain-derived neurotrophic factor signaling decreased local translational of mRNA of synaptic proteins in hippocampal synaptosomal preparation by a recombinant extracellular domain of human tropomyosin-related kinase receptor type B fused to the Fc region of human IgG (TrkB-Fc chimera) in naïve rats. (*A*) Microinjection of Trkb-Fc chimera (2 μg × 7 days) significantly decreased phosphorylation of eukaryotic translation initiation factor 4E (eIF4E; t = 4.817, DF = 10, two-tailed *P* = 0.0007), phosphorylated 70-kDa ribosomal protein S6 kinase (t = 3.3, DF = 10, two-tailed *P* = 0.0082), and S6 (t = 3.7, DF = 10, two-tailed *P* = 0.0042) in the hippocampal synaptosomal preparation. (*B*) Microinjection of Trkb-Fc chimera (2 μg × 7 days) also decreased the expression of GluR1 (a postsynaptic protein; t = 4.6, DF = 10, two-tailed *P* = 0.001), SV2 (a presynaptic protein; t = 2.9, DF = 10, two-tailed *P* = 0.0154), and calcium/calmodulin-dependent protein kinase II α (CaMKIIα; t = 8.1, DF = 10, two-tailed *P* < 0.0001) in the hippocampal synaptosomal preparation. n = 6 rats in each group. ***P* < 0.01; ****P* < 0.001. Data represent mean ± SD.

notion that the reduction of the hippocampal BDNF contributes to the cognitive deficiency in the adult rodent with previous exposure to neonatal anesthesia.

BDNF exhibits remarkable potency as a translational enhancer that modulate the function of translational machinery in the dendritic spines, thus contributing to the induction and maintenance of synaptic plasticity in the central neurons.[21,51,58] In general, synaptic translation is initiated with the formation of the eIF4F complex (composed of eIF4E, eIF4A, and eIF4G), followed by recruitment of the ribosome, the mRNA molecule, and the elongation factors. The formation of this complex and induction of translation activity is promoted by phosphorylation of eIF4E-binding proteins.[59] Phosphorylation of ribosomal subunit S6 by p70S6K also correlates with the translation of synaptic mRNAs.[16,60,67] In this study, a significantly decreased phosphorylation of translational components (eIF4, p70-S6 kinase, and S6) indicating a reduced function of local translational machinery in the hippocampal CA1 area due to the reduction of the hippocampal BDNF in the adult rodent with neonatal anesthesia. The reduced expression of synaptic proteins (*e.g.*, SV2, GluR1, and CaMKIIα) was also observed in the hippocampal CA1 in the modeled rodents and in naïve animals that received TrkB-Fc chimera. Figure 9 depicts the important components in the signaling pathway affected by isoflurane anesthesia in neonatal rats that result in the loss of synaptic plasticity and cognitive impairment in the adult animal. Our results suggested that neonatal anesthesia-induced BDNF reduction led to insufficient drive for the local translation of mRNA of synaptic proteins, thus contributing to the impaired synaptic plasticity and cognition in the rodents with neonatal anesthesia.

Recent reports have illustrated the profile of epigenetic modulation of *Bdnf* expression in different physiological and several pathological scenarios.[68] The human *Bdnf* gene contains 11 exons and 9 promoters with alternative splices.[68] Although altered histone acetylation in the promoter regions may significantly modulate the transcription of *Bdnf* exons (fig. 9), promoter regions of *Bdnf* exons contain abundant CpG sites, which are subjected to the cytosine methylation catalyzed by the DNMTs.[68] This epigenetic modification of *Bdnf* transcription may largely change the expression of BDNF and contribute to the pathogenesis of several neurological disorders. For instance, increased histone H4 acetylation in the promoter upregulated the *Bdnf* exon I and IV transcripts was associated with the extinction of conditioned fear in the rodent.[69] Increased occupancy of H3K27me3 in the promoter region was associated with a reduction of BDNF expression in the ventral tegmental area induced by cocaine.[70] Increased promoter methylation of *Bdnf* exons, along with the decreased expression of BDNF, was reported in several brain regions including the hippocampus in patients with neurological disorders such as schizophrenia,[71] depression, and anxiety.[72] In this study, significantly decreased histone H3 acetylation and increased cytosine methylation were observed in the promoter region of *Bdnf* exon IV, which may contribute to the long-lasting hippocampal BDNF reduction induced by neonatal anesthesia.

Several transcriptional factors such as MeCP2, HDAC2, and DNMT1 exhibit the potency to alter the chromatin structure of *Bdnf* genes, thus modulating the transcription and expression of BDNF (fig. 9). Increased MeCP2 and HDAC2 occupancy in the promoter of *Bdnf* exon IV may substantially reduce the expression of BDNF in the hippocampus in the

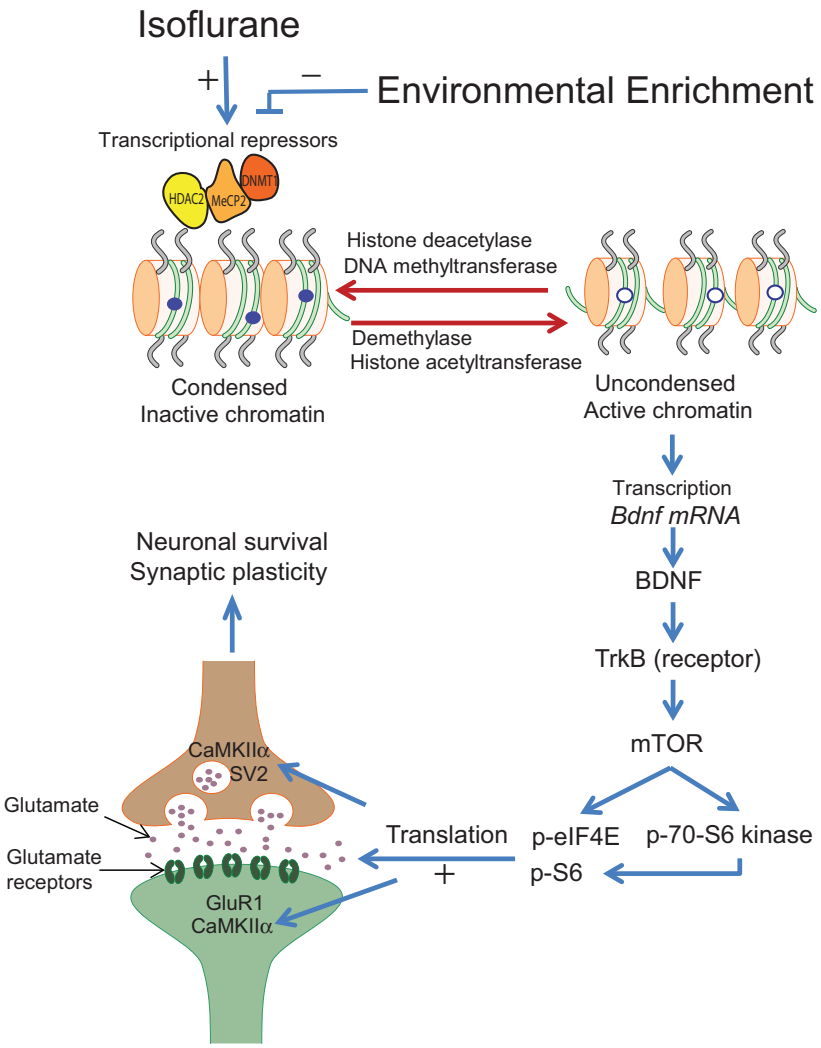

Fig. 9. Schematic representation of the signaling pathway affected by isoflurane anesthesia in neonatal rats on the expression of brain-derived neurotrophic factor (BDNF) resulting in cognitive dysfunction in the adult animal. Inside the nucleus, the genetic material (*green line*) is organized into higher order structures, termed chromatin, by wrapping around small histone proteins (*orange cylinders*). Methylated cytosines (*blue circles*) and deacetylated histones represent repressive modifications that interfere with binding sites of transcription factors, thereby inhibiting induction of gene expression (the chromatin is condensed and inactive). In addition, methylated DNA–binding proteins such as methyl-cytosine-phosphate-guanine–binding protein 2 (MeCP2) may repress gene transcription (*Bdnf* in this model) through their association with a nuclear corepressor complex that possesses histone deacetylase 2 (HDAC2) activity. DNA methyltransferase 1 (DNMT1) maintains a stable cytosine methylation state. In permissive modifications, DNA of a gene promoter is unmethylated (*white circles*; the chromatin is uncondensed and active), and the *Bdnf* gene can be expressed. Released BDNF stimulates tropomyosin-related kinase receptor type B (TrkB) and downstream mammalian target of rapamycin (mTOR) signaling, which increases phosphorylated eukaryotic translation initiation factor 4E (eIF4), phosphorylated 70-kDa ribosomal protein S6 kinase (p70-S6 kinase) and phosphorylated ribosomal protein S6 resulting in increased translation of transcripts, including expression of GluR1 (a postsynaptic protein), SV2 (a presynaptic protein), and calcium/calmodulin-dependent protein kinase II α (CaMKIIα, a ubiquitous protein), which play important roles in promoting synapse formation and maturation in the developing brain as well as regulation of synaptic transmission in the hippocampal and cortical neurons. Our data show that isoflurane anesthesia in P7 rats resulted in a substantial reduction of hippocampal BDNF resulting from the transcriptional factors–mediated epigenetic modification in the promoter region of *Bdnf* exon IV. This BDNF reduction led to insufficient drive for the synthesis of synaptic proteins, resulting in hippocampal synaptic and cognitive dysfunction. We also found that exposure to environmental enrichment can trigger histone acetylation and cytosine demethylation, thereby rendering the chromatin more permissive to *Bdnf* transcription. Environmental enrichment has pronounced effects in mitigating isoflurane-induced impairments on *Bdnf* transcription, hippocampal synaptic plasticity, and cognitive functions.

rodent.[73,74] Increased DNMT1 activity was associated with the BDNF reduction in the mice model of prenatal stress–induced schizophrenia.[20] The synergistic action among these transcriptional repressors seems to modulate the expression of specific genes and contributes to cognitive decline in several neurological disorders.[37] MeCP2 binds to the methylated

CpG sites in the promoter region and recruits the histone deacetylases, including HDAC2, to remove the acetyl group from the histones, thereby rendering the chromatin more repressive to the target genes transcription (fig. 9).[75,76] MeCP2 may form the complexes with DNMT1 to maintain DNA methylation in the genome.[57] In this study, significantly increased interaction between MeCP2 and HDAC2 or DNMT1 as well as the occupancy of these repressors in the promoter region of *Bdnf* exon IV was observed in the hippocampal CA1, which was associated with increased cytosine methylation and decreased histone H3 acetylation in the promoter region in the modeled animal. These adaptations of transcriptional repressors potentially contributed to the hippocampal BDNF reduction. It is important to note that the activity of HDAC2, but not histone deacetylase 1 or histone deacetylase 3, was found to be upregulated in the hippocampal CA1 in rodent models of memory deficiency.[56] Furthermore, alterations of acetylation/methylation of histone H3 in the *Bdnf* promoter region regulate the expression of BDNF in several neurological disorders.[77,78] For these reasons, we chose to study the changes in histone H3 and HDAC2 in P7 rats after isoflurane anesthesia.

This study also demonstrated that EE has a potential therapeutic effect on the memory deficiency induced by neonatal anesthesia. For several decades, numerous studies have reported that exposure to EE can substantially increase the synaptogenesis and spine formation in CA1 neurons of the hippocampus and cortex[23,24] and enhance the glutamatergic synaptic strength and LTP triggered by theta-burst stimulation in the hippocampus and cortex.[25] EE can overcome learning deficits in genetically challenged mice,[23] enhance recovery after lesions in adult animals, and markedly improve learning ability as evidenced by stronger freezing behavior in the contextual fear condition test[26] and better performance in the Morris water maze test in rodents lacking maternal care.[27] In this study, we also found that EE restored the hippocampal synapses and spine density, improved the function of synaptic translational machinery and the synthesis of synaptic protein, and ameliorated the hippocampal synaptic and cognitive impairments induced by neonatal anesthesia. One of the limitations of this study is that the present model may not be necessarily relevant in the context of routine care of human neonates as the pups were not ventilated and normocapnia was not controlled during anesthesia.

Previous studies have also suggested that the exposure to EE may induce a change in BDNF signaling, thus contributing to the improved synaptic plasticity and cognitive function.[79,80] Prolonged exposure to EE was associated with an increase in cerebellar and hippocampal production of BDNF in the rodent.[23,24,81] EE restored the hippocampal BDNF and improved the behavioral performance in the novel object recognition and Morris water maze tests in the rats with chronic cerebral hypoperfusion.[82] Exposure to early enrichment can lead to high levels of histone acetylation at the *Bdnf* gene at adulthood, thus facilitating the expression of BDNF.[83,84] In this study, we found that EE mitigated the neonatal anesthesia-induced epigenetic modification in the promoter region of *Bdnf* exon and restored the hippocampal expression of BDNF in the modeled rodent. This modulation of BDNF signaling underlies the therapeutic effect of EE on the hippocampal synaptic and cognitive dysfunction induced by neonatal anesthesia.

Taken together, neonatal exposure to isoflurane anesthesia significantly reduced the expression of BDNF *via* epigenetic mechanisms, thus suppressing the local translation of synaptic mRNAs in the hippocampal CA1, which substantially contributed to synaptic dysfunction and memory deficiency during rat adulthood. These impairments were reversed by the exposure to an enriched environment.

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Competing Interests

The authors declare no competing interests.

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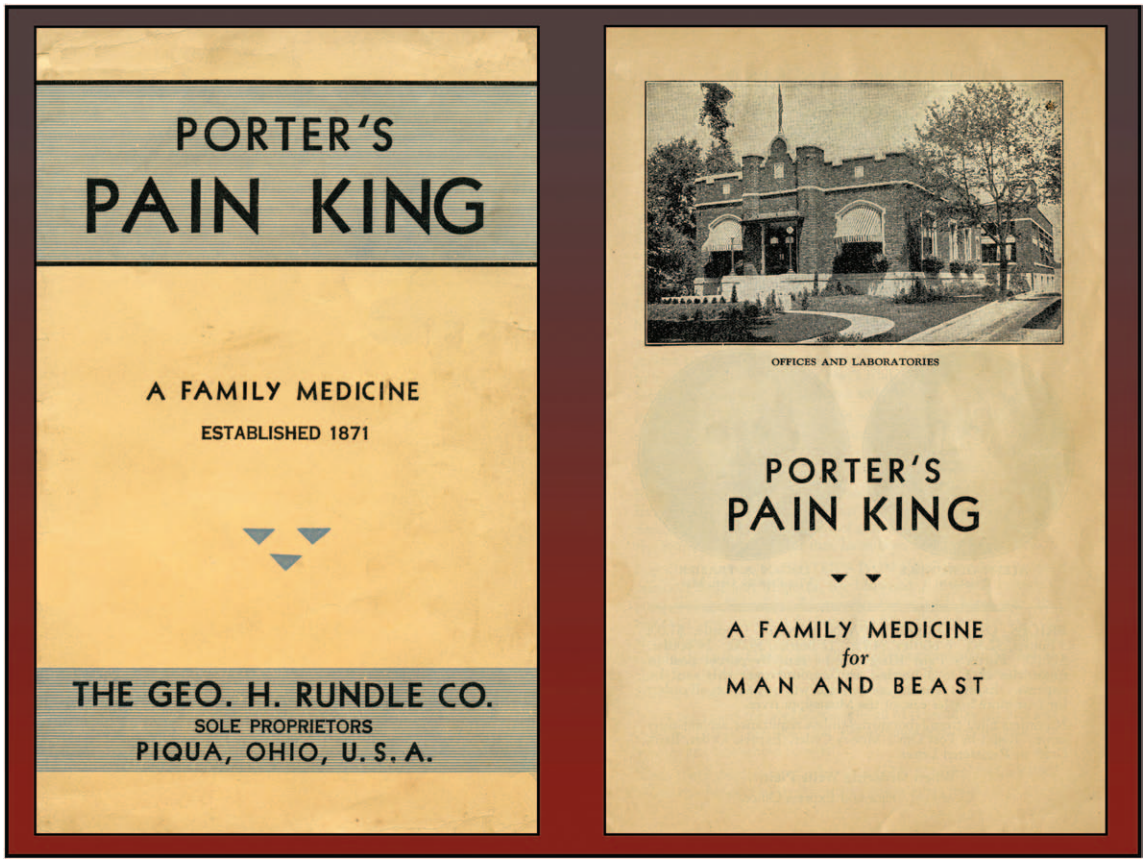
ANESTHESIOLOGY REFLECTIONS FROM THE WOOD LIBRARY-MUSEUM

Porter's Pain King: Ether in Alcohol "for Man and Beast"



According to their brochure cover (*left*), the George H. Rundle Company owned and distributed "a Family Medicine for Man and Beast"— Porter's Pain King. The brochure also depicts the company's "Offices and Laboratories" (*right*), which were headquartered in Piqua, Ohio. Labeled as capable of relieving "Colds, Nervous and Sick Headache," bottles of this "Family Liniment" were also touted for curing "Rheumatic and Neuralgic Pains, Toothache, Backache, Sprains, Bruises, and Burns." Relief by this remedy was likely temporary but was likely aided by its "ETHER 12 MINIMS PER OUNCE" and certainly assisted by its "ALCOHOL [content of] 63%." As a proper remedy for the family farm, Porter's Pain King could be administered to family members, household pets, and even barnyard animals. (Copyright © the American Society of Anesthesiologists, Inc.)

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